

Transfection of bovine spermatogonial stem cells *in vitro*

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Summary

Spermatogonial stem cells (SSCs) are the only stem cells in adults that can transfer genetic information to the future generations. Considering the fact that a single SSC gives rise to a vast number of spermatozoa, genetic manipulation of these cells is a potential novel technology with feasible application to various animal species. The aim of this study was to evaluate enhanced green fluorescent protein (EGFP) gene transfection into bovine SSCs via liposome carrier and assess the best incubation day in uptake exogenous gene by SSCs. Transfection efficiency of EGFP gene with lipofectamine 2000 was determined in days following each three day of transfection (day 4, 6 and 8 of the culture) by fluorescent microscope. Results showed that the transfected cells through lipofection increased significantly ($P < 0.05$) in each three days of transfection in comparison with those of the control groups. The transfected SSCs were higher in comparison with those of the free exogenous gene carrier groups ($P < 0.05$). In comparison with these three days, the rate of infected cells was higher when transfection proceeds at day four. It was concluded that lipofectamine can be used safely for direct loading exogenous DNA to SSCs particularly during the fourth day of culture.

Key words: EGFP, Lipofection, Spermatogonial stem cells

Introduction

Spermatogenesis is an organized process consisting of complex sequential steps of cell proliferation and differentiation on the basis of spermatogonial stem cells (SSCs). Spermatogonial stem cells are the only cells in postnatal mammals that self-renew and transfer genes to the next generation (Kubota and Brinster, 2006). Stem cells are the ones that can self-renew as well as producing one or more mature type cells during differentiating process. So the manipulation of male germ line becomes possible through the manipulation of these cells. This feature allows us to manipulate and alter the phenotype of offspring, including the correction of mutations that cause diseases such as autism, diabetes, Down syndrome and heart disease. Also, these cells are directly involved in fertility. Disorders in the course of these cells have negative impacts on spermatogenesis and reduce fertility and infertility (Brinster and Avarborck, 1994; Horfman, 2008; Kubota and Brinster, 2008).

Transgenesis includes the transfer of foreign DNA sequence into the genome of a specific specimen in such a way that those sequences become detectable in more cells, then are transmitted to the next generation. Transgenesis in farm animals is used in order to improve production traits and evolution of transgenic classes for production of pharmaceutical proteins, medical and tissue transfer (Niu and Liang, 2008; Xiang-Yang, 2011).

Germ-cells gene transfer methods include viral and non-viral methods. Up to now, vectors based on

retrovirus, lentiviruses and adeno/herpes virus have been used for gene transfer. Generally, recombinant viral vectors for gene therapy are produced by minor shifts of viral genes with therapeutic genes. Although a viral vector can infect cells with relatively good efficiency, the risk of viral gene transcription and insertion of these genes into the host genome still exist. Also, the production of viral particles requires high expertise (Masahito *et al.*, 2003; Whitelaw *et al.*, 2008). Non-viral vectors, such as plasmids that can be transported into cells via liposomes or electroporation are available. These are *in vivo* safely and facile to use. Non-viral vectors successful transfection, especially with liposomes has been used in transplantation. The best candidates as the target for non-viral gene transfer are cells with efficient potential of proliferation and self-renewal. Thus, embryonic and adult stem cells are considered as a source of non-viral mechanisms (Ogach *et al.*, 1998; Lai *et al.*, 2008).

The basis of this method is the electric charge, the nature of lipid globules (liposomes) and their ability to pass through cell membranes and enter them. In fact, in this way we insert the designed gene into the lipid-based globules and then put it close to the host cells, therefore liposome will import into the host cells and take our candidate gene structure into the cells. The exact mechanism is still unclear. The cell type, the type of liposome, the specificity and the likelihood of liposomes to target cells are very important (Felgner *et al.*, 1987).

The aim of this study was to evaluate enhanced green fluorescent protein (EGFP) gene transfection into bovine

SSCs with liposome carrier and assess the best incubation day in uptake exogenous gene by SSCs.

Materials and Methods

Animals and testicular biopsy

The present study was approved by the Animal Ethics Committee, University of Tehran to obtain testicular tissue, Holstein calves (n=8), aged 3-5 months were subjected to testicular biopsy as previously described (Izadyar *et al.*, 2002). Briefly, testicular biopsy was performed under sedation with xylazine (0.2 mg/kg, Alfasan Holland) and local anesthesia with lidocaine (Aburiah Pharma Co., Iran). Following incision, the testicular tissue was obtained and placed into a 15 ml tube containing Dulbecco minimal essential medium (DMEM, Gibco, England) with 10% fetal bovine serum (FBS, Sigma-Aldrich, US) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin, Gibco). The specimen was subsequently transferred on ice to the laboratory within 2 h.

Cell isolation

Cell isolation was implemented using a two-step enzymatic isolation procedure, as previously described by Izadyar *et al.* (2002) with minor modification. In brief, the testicular tissue was washed three times in DMEM containing antibiotics and was minced into small pieces using sterile scissors. The minced testicular tissue was incubated in DMEM containing 1 mg/ml collagenase (Sigma-Aldrich, US), 1 mg/ml hyaluronidase (Sigma-Aldrich, US), 1 mg/ml trypsin (Sigma-Aldrich, US) and 5 g/ml DNase (Fermentas, Germany) at 37°C in a shaker incubator with 80 cycles per min for approximately 60 min. The digested testicular tissue was washed three times with DMEM and the supernatant was disposed after each washing, leading to isolation of seminiferous tubules. During the second step of enzymatic digestion, the seminiferous tubules were incubated at 37°C in DMEM containing 1 mg/ml collagenase, 1 mg/ml hyaluronidase and 5 g/ml DNase until disintegration of the seminiferous tubules and separation of the constituent cells. Individual cells were isolated from the remaining tubule fragments by centrifugation at $30 \times g$ for 2 min. Following filtration through 77 and 55 µm nylon filters, the cells were pelleted. The pellet was re-suspended in the DMEM containing antibiotics and 10% knock-out serum replacement (KSR, Gibco, England).

Cells were seeded in DMEM with antibiotics and 10% FBS, and cultured in a 24-well cell culture-treated plate (TPP®, Switzerland) at a concentration of 300,000 cells/well ($88.2 \pm 3\%$ viability) and incubated at 37°C in a humidified atmosphere with 5% CO₂.

Viability assay

Viability of the primary isolated cells was evaluated by trypan blue staining and the total number of the cells present in suspension was determined using a hemocytometer under a light microscope.

Evaluation of SSCs colonization

Spermatogonial colonies in each well were counted using a differential interference contrast microscope (Olympus, IX71® inverted microscope) at days 4, 6 and 8.

Immunofluorescent staining against OCT-4 and vimentin

For immunostaining, cells (from the cell suspension acquired from step-2 of enzymatic digestion) were plated into 4-well chamber slides with each chamber containing 50,000 cells in DMEM with antibiotics and 10% FBS and incubated at 37°C in a humidified atmosphere with 5% CO₂. Cultures were stopped and fixed after 5 days. Primary and secondary antibodies used for detecting OCT4 in SSCs were rabbit anti-OCT4 (polyclonal antibody ab18976, Abcam, Cambridge, UK) and FITC-conjugated goat anti rabbit IgG. Primary and secondary antibodies used for observing vimentin in Sertoli cells were mouse anti-vimentin (monoclonal antibody ab8069, Abcam, Cambridge, UK) and fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG. Cell staining was performed according to the procedure previously described by Qasemi-Panahi *et al.* (2011).

Plasmid extraction

In this study the EGFP plasmid construction (pEGFPN1, 5.4 kb) used for our experiments contained the CMV promoter and the EGFP gene (Fig. 1). Plasmids were extracted using Plasmid Mega Kit (Qiagen) and the *Stu*I restriction enzyme (Takara, Japan) was used to prepare linearized plasmid following the manufacturer's instructions and digestion efficiency was checked by 1% agarose gel electrophoresis.

Short-term treatment with trypsin

Before the gene transfer process, it is necessary to spread SSCs individually as much as possible and reduce their accumulation. Therefore, the combination of mechanical and chemical digestion should be performed the day before transfection. After removing the cell from the culture medium and washing with serum-free culture or PBS, 0.5 to 1 ml trypsin 0.5% containing EDTA (*in vitro* gen) was added to the cells. Then the cells were incubated for 3 min. During this time, by the mechanical tapping on the bottom of the culture dish, the reduction of density and relative isolation of cells was facilitated. Upon removal of cells from the base, in order to neutralize the trypsin, FBS was added. In this stage, single cells appeared.

Experimental groups

In this survey there are 3 experimental groups as follow:

- Group 1: Co-culture of spermatogonial cells with testis somatic cells (control)
- Group 2: Co-culture of spermatogonial cells with testis somatic cells along with DNA (with DNA)
- Group 3: Co-culture of spermatogonial cells with testis somatic cells along with DNA + lipofectamine complex

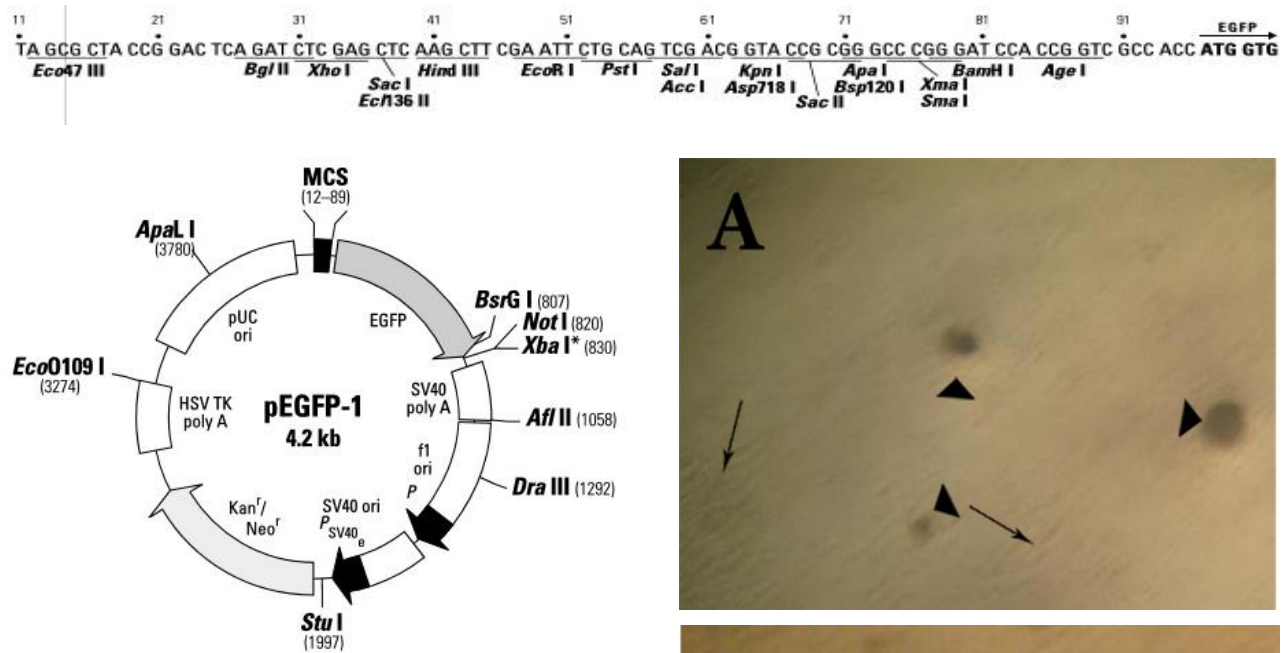


Fig. 1: EGFP plasmid gene mapping

(with DNA + lipofectamine)

GFP gene transfer using liposomal carriers (lipofection)

In this study, in order to transfer gene into the bovine SSCs using liposomal carriers, lipofectamine 2000 DNA transfection reagent (invitrogen) was used. Twenty four h before the transfection, cell suspension was initially confirmed in the log growth phase. In summary, in order to perform lipofection method, 4 μ L of lipofectamine diluted in 25 μ L DMEM and 2 μ g of DNA (plasmid) diluted in 25 μ L DMEM. Then diluted lipofectamine was added to the diluted DNA in a sterile micro-tube and after 5 min incubation at 37°C 56 μ L of DNA-lipofectamine complex was added to each well. In this study to evaluate the best day on which the highest rate of transfected cells could be obtained, transfection rate was performed in days 4, 6 and 8 of culture, and the results were followed for up to 3 days following the transfection. In order to determine the transferred gene in SSCs, cells were investigated under an inverted fluorescent microscope (NIKON ECLIPSE E600) using UV radiation of wavelengths 460-500 nm (blue filter). If the transfer is successful, green fluorescent will be seen, confirming that gene transfer is approximately successful.

Results

Isolation and identification of spermatogonial and sertoli cells

The cell population obtained from seminiferous tubules of 3-5 month-old calves testis contained mostly two cell types with different morphological characteristics. The first cell type proliferated and created a monolayer of cells, whereas the other cell type created a

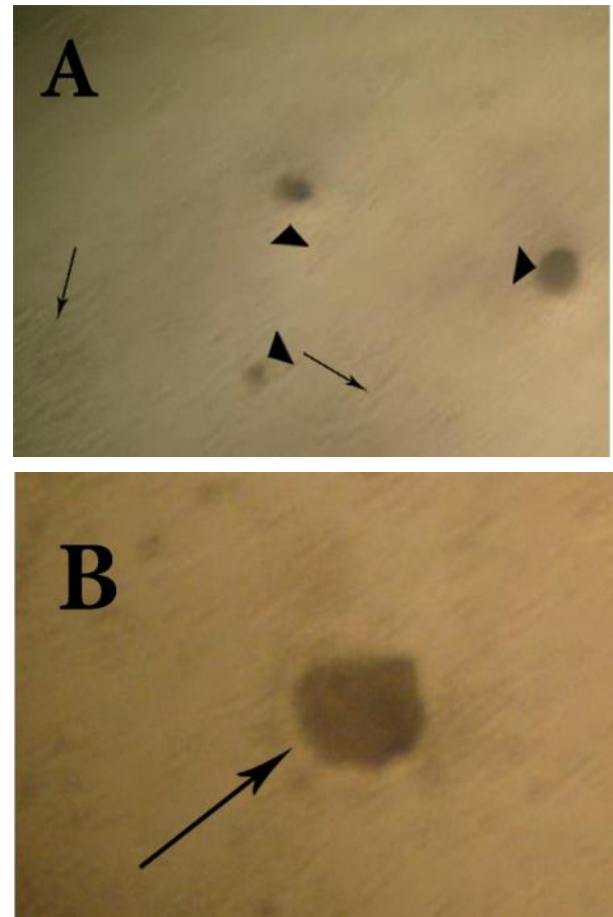


Fig. 2: Spermatogonial stem cells morphology. A) Sertoli cells that created a monolayer of cells (arrow), some little spermatogonial derived colonies were seen (triangle), and B) The morphology of a spermatogonial derived colony that formed from co-cultured spermatogonial cells on a monolayer of sertoli cells, ($\times 200$)

colony after proliferation occurred (Fig. 2A). These colonies have morphological characteristics of bovine SSCs colonies; they were round, with distinct margin and with brown color that were located on sertoli cells layer (Fig. 2B).

Immunofluorescent staining

At day 5 of culture, vimentin was detected in sertoli cells which developed a feeder monolayer (Fig. 3), and OCT4 was demonstrated in the spermatogonial colonies (Fig. 4).

GFP gene transfer to the spermatogonial colonies using lipofection method

In day 4, 6 and 8 of culture the number of infected colonies was significantly higher than the control group. The significant difference was also observed in the group with no carrier in order to transfer foreign gene to SSCs

($P < 0.05$) (Tables 1, 2 and 3). On the other hand, on days 4 and 6, a number of colonies in the group without carrier for foreign genes, have also been contaminated (Tables 1 and 2). While on day 8 no spontaneous uptake was seen among colonies (Table 3). When transfection was performed on day 4, the colonies infection rate was higher than on days 6 and 8 (Table 1). Green fluorescent represented EGFP gene transfer to the spermatogonial colonies (Fig. 5).

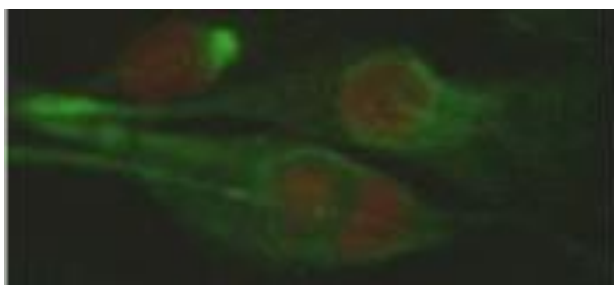


Fig. 3: Immunofluorescent staining of sertoli cells for vimentin (green) at day 5 of culture

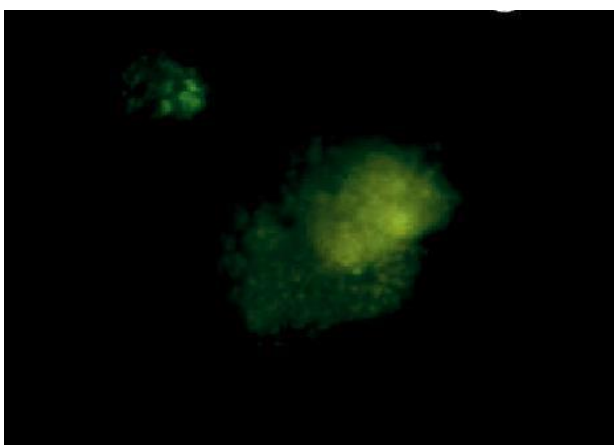


Fig. 4: Immunofluorescent staining of SSCs for OCT4 (green) at day 5 of culture

Table 1: The percentage of penetration of DNA to bovine spermatogonial colonies using lipofectamine when the experiment began on day 4 (\pm SD)

Groups	Days		
	5	6	7
Control	0 ^a	0 ^a	0 ^a
With DNA	0 ^a	0.8 \pm 0.8 ^a	1.6 \pm 1.6
With DNA + lipofectamine	29.8 \pm 7 ^b	34 \pm 6.6 ^b	37 \pm 6.5 ^b

^{a, b} Different letters in the columns representing significant difference ($P < 0.05$)

Table 2: The percentage of penetration of DNA to bovine spermatogonial colonies using lipofectamine when the experiment began on day 6 (\pm SD)

Groups	Days		
	7	8	9
Control	0 ^a	0 ^a	0 ^a
With DNA	0 ^a	1 \pm 1 ^a	1 \pm 1 ^a
With DNA + lipofectamine	12.2 \pm 3 ^b	14.8 \pm 3.1 ^b	18.4 \pm 3.6 ^b

^{a, b} Different letters in the columns representing significant difference ($P < 0.05$)

Table 3: The percentage of penetration of DNA to bovine spermatogonial colonies using lipofectamine when the experiment began on day 8 (\pm SD)

Groups	Days		
	9	10	11
Control	0 ^a	0 ^a	0 ^a
With DNA	0 ^a	0 ^a	0 ^a
With DNA + lipofectamine	2.6 \pm 4 ^b	2.4 \pm 0.2 ^b	3.4 \pm 0.4 ^b

^{a, b} Different letters in the columns representing significant difference ($P < 0.05$)

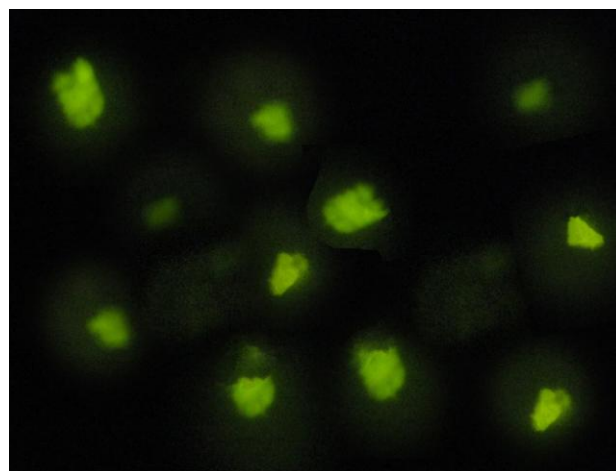


Fig. 5: Green fluorescent represented EGFP gene transfer to the spermatogonial cells at day 6 of culture

Discussion

Spermatogonial stem cells are particularly important in cattle, which have a long generation interval compared to laboratory animals and smaller species of livestock. A system in which genes could be delivered to cattle through SSCs would considerably shorten the time to obtain transgenic animals (Khaira *et al.*, 2005; Ryu *et al.*, 2005; Hermann *et al.*, 2007).

According to Izadyar *et al.* (2002), great population of sertoli and spermatogonial cells can be achieved from 3–5-month-old calves. At this age, the testes have a great pool (65-87%) of type A spermatogonia cells.

Typically, a dense population of SSCs with high purity of about 1×10^6 stem cells per gram of testicular tissue have the survival rate $>80\%$. Survival rate obtained in this study is comparable to studies of neonatal mouse (Bellue *et al.*, 1977), rat (Morena *et al.*, 1996; Anway *et al.*, 2003), pork (Dirami *et al.*, 1999) and calves (Izadyar *et al.*, 2002).

In this study, among the cells isolated from the 3 to 5 month-old calves seminiferous tubules, there were two kinds of cells with various immunocytochemical features, similar to sertoli cells and type A spermatogonial stem, respectively. This finding is consistent with Kourji *et al.* (2007) reports that demonstrated the immunocytochemical features of sertoli and SSCs in mice.

For identification of sertoli cells, we used vimentin immunocytochemical staining (Sigma Chemical Co., St.

Louis, Mo., USA) (Anway *et al.*, 2003; Tajik *et al.*, 2012). Colonies had morphology of bovine SSCs (Izadyar *et al.*, 2000). So we concluded that colonies were derived from the SSCs.

In this study, the lipofectamine 2000 was used in order to carry foreign EGFP gene to the SSCs. The results showed that the uptake of foreign gene by liposomal carrier in all three days of lipofection increases significantly compared with the group with no carriers (Tables 1, 2 and 3). The results of this study showed that the logarithmic growth phase peak is significantly important to determine the day with the highest rate of plasmid transfection into spermatogonial colonies. This finding is in agreement with the study of Takahashi *et al.* (2007) using the adenovirus vector in mice. However, on day 4 the highest transfection rate of DNA without carrier was observed in the SSCs.

On day 6 of transfection, DNA transfection rate without carrier was constant and observed in only two days. These findings are in contrast with the survey on the uptake of DNA in mouse sperm without carrier (Younezawa *et al.*, 2002).

On day 8 of transfection, percentage of infected cells with the foreign gene was very low, moreover, there was not any spontaneous DNA uptake in spermatogonial cells. This indicate reduction in the number and viability of spermatogonial cells (Felgner *et al.*, 1987).

Studies on the production of transgenic SSCs are based on *in vivo* studies, i.e. the foreign gene has been injected into the testes (testis-mediated gene transfer TMGT) (Sciamanna, 2002). In this regard, Ogachi *et al.* in 1998 injected GFP marker gene into the testes of 7-day-old young mice for further investigation and possible limitations of TMGT method. Then at weeks 6, 12 and 24, the male mice mated with the female mice and expression of EGFP was evaluated in the F1 transgenic pups (Hui Ming *et al.*, 2011).

Nagona *et al.* (2001) showed that although retroviral vectors are used as effective gene vector into a wide range of cells, the germ stem cells after the birth are resistant to infection with these viruses. In addition, expression of genes inserted into different types of stem cells such as embryonic stem cells or hematopoietic stem cells are often very weak or turned off. They showed that *in vitro* gene transfer by retroviral vector into immature and mature mice SSCs, results in stable integration and expression of a transgene only in 2-20% of the stem cells (Nagona *et al.*, 2001).

This is the first study to examine the insertion of gene into the SSCs *in vitro*, using liposomal carriers.

This method is based on electric charge, the nature of lipid globules (liposomes), and their ability to pass through the cell membranes and enter them. In fact, in this way we insert the gene structure into the lipid globules, and then put it on the side of the host cell. Then the liposome and the gene structure are inserted into the host cell. The exact mechanism is still unclear. Cell type, type of liposome, the specificity and the likelihood of liposomes to target cells are very important in the success of this procedure (Felgner *et al.*, 1987; Garrett *et*

al., 1997).

It is said that the use of liposomes for cell transfection can protect DNA against endogenous nucleases. Avoid direct interaction between foreign DNA and the cell membrane allows foreign DNA reach to the nucleus without pulling the trigger of apoptosis (Garrett *et al.*, 1997).

Overall, the findings of this study suggest that the use of liposomal carriers is a low-risk, secure and high performance method to transfer foreign genes to the SSCs. It also enhances hopes of providing differentiation conditions against producing transgenic sperms of SSCs *in vitro*.

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